



Immunoglobulin-binding Sites of Human Fc α RI (CD89) and Bovine Fc γ R2 Are Located in their Membrane-distal Extracellular Domains[#]

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SUMMARY

To localize the immunoglobulin (Ig)-binding domains of the human myeloid IgA Fc α receptor (Fc α RI, CD89) and the bovine Fc γ 2 receptor (bFc γ 2R), chimeric receptors were generated by exchanging comparable regions between these two proteins. Fc α RI and bFc γ 2R are highly homologous and are more closely related to each other than to other human and bovine FcRs. Nevertheless, they are functionally distinct in that Fc α RI binds human IgA (hIgA) and not bovine IgG2 (bIgG2), whereas bFc γ 2R binds bIgG2 and not hIgA. Fc α RI and bFc γ 2R possess extracellular regions consisting of two Ig-like domains, a membrane-distal extracellular domain (EC1), a membrane-proximal EC domain (EC2), a transmembrane

region, and a short cytoplasmic tail. Chimeras, constructed by exchanging complete domains between these two receptors were transfected to COS-1 cells and assayed for their ability to bind hIgA- or bIgG2-coated beads. The results showed that the Ig-binding site on both Fc α RI and bFc γ 2R is located within EC1. Supporting this observation monoclonal antibodies that blocked IgA-binding to Fc α RI were found to recognize epitopes located in this domain. In terms of FcR-Ig interactions characterized thus far, this location is unique and surprising because it has been shown previously that leukocyte Fc γ Rs and Fc ϵ RI bind Ig via sites principally located in their EC2 domains.

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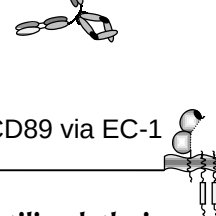
INTRODUCTION

Immunoglobulin (Ig) Fc receptors (FcRs) expressed on phagocytic cells provide a crucial link between the humoral and cellular branches of the immune system. Ligation of FcR by antigen-bound Ig leads to cellular activation and triggering of powerful effector mechanisms (1,2).

In humans and other mammals, IgA predominates in mucosae and, furthermore, comprises a substantial proportion of the circulating Ig pool. At mucosal surfaces IgA provides a first-line protective function, termed immune exclusion, whereby it inhibits microbial colonization on epithelial cells and penetration of harmful antigens. In addition, the protective function of IgA both in mucosa and in the circulation maybe reinforced by interaction of IgA-complexed antigens with the myeloid Fc α RI (CD89) (3,4). Fc α RI is expressed on monocytes, macrophages, polymorphonuclear granulocytes, and eosinophils, and its crosslinking triggers a variety of immunological effector functions, including phagocytosis, antibody-dependent cellular toxicity, and release of inflammatory mediators and cytokines. (3-6).

Among FcRs characterized until now, Fc α RI is most closely related to the bovine Fc receptor for IgG2 (bFc γ 2R) expressed on monocytes and granulocytes. In fact, these two FcRs are more closely related to each other than to any known human or bovine FcRs (7). More recently, it has been shown that Fc α RI and bFc γ 2R are members of a new gene family that apparently evolved from a common ancestral gene. Other human genes belonging to this family include the natural killer cell inhibitory receptors (KIRs), the Ig-like transcripts (ILTs), the leukocyte and monocyte/macrophage Ig-like receptors (LIRs, MIRs), LAIR-1, and HM18 (8-13). These genes are located close to the Fc α RI gene within the so-called leukocyte receptor complex on chromosome 19q13.4 (13-15). Several murine members of the same gene family, gp49B1 (a structural homologue of human KIRs), and the paired Ig-like receptors A and B (PIR-A and PIR-B) have also been described (16-18).

Fc α RI and bFc γ 2R are both transmembrane glycoproteins composed of two extracellular (EC) Ig-like domains (EC1 and EC2), a transmembrane region containing a charged arginine residue, and a short cytoplasmic tail devoid of signaling motifs (7,19). Signal transduction via Fc α RI is mediated via the FcR γ chain, which associates with Fc α RI through the charged arginine residue within its transmembrane domain but does not affect its affinity for IgA (20-23). Despite the high level of amino acid identity (41%) within the EC and transmembrane regions of Fc α RI and bFc γ 2R, these two receptors are functionally quite distinct in that Fc α RI binds IgA but not bIgG2, whereas bFc γ 2R binds bIgG2 but not IgA.



Therefore, to map the ligand-binding domains of these two FcRs we utilized their high degree of identity and exchanged homologous regions between them. Based on knowledge of interactions between other two-domain FcRs (Fc γ RII, Fc γ RIII and Fc ϵ RI) with their respective ligands (IgG or IgE), we expected the Ig-binding sites to be located within the membrane-proximal EC2 domain (24-30). Surprisingly, however, our results demonstrated that the ligand-binding region of both Fc α R and bFc γ 2R is located in their membrane-distal EC1 domains. In part, this finding probably reflects the evolutionary development of Fc α RI and bFc γ 2R from an ancestral gene distinct from the putative Fc γ R/Fc ϵ R precursor.

MATERIALS AND METHODS

Cell culture

COS-1 cells were maintained in DMEM (Bio Whittaker) supplemented with 10% FCS, 1mM L-glutamine, and 50 μ g/ml gentamycin (Life Technologies, UK). The murine IIA1.6 B cell line that coexpresses Fc α RI and the FcR γ -chain has been described (21).

cDNAs and construction of chimeric FcRs

cDNAs encoding the complete Fc α RI coding region and a mutant cDNA encoding a soluble form of Fc α RI were gifts from Dr. C. Maliszewski (Immunex Corp., Seattle WA) (19,31). cDNA for bFc γ 2R has been described previously (7). Chimeric cDNAs were constructed by overlap extension PCR (21). Although the genomic structure of bFc γ 2R is unknown, the high homology to Fc α RI allowed us to infer intron-exon boundaries because amino acid residues that link the Fc α RI exons are identical to those at comparable positions in bFc γ 2R (Figure 1). Primers were thus designed to allow the fusion of exons at these residues. To construct the bEC1⁽¹⁻⁵⁰⁾-Fc α RI mutant, primers were designed to allow the fusion of the first 50 amino acids of bFc γ 2R to Fc α RI at isoleucine 50; both Fc α RI and bFc γ 2R have isoleucine residues at this position, which lies almost exactly in the middle of the EC1 domains. The integrity of all chimeric cDNAs was confirmed by sequence analysis. Chimeric FcR cDNAs were cloned into the pCDNA3 mammalian expression vector (Invitrogen, The Netherlands) prior to transfection.

Transfections

COS-1 cells were transiently transfected with 2 μ g chimeric FcR cDNA constructs by means of Eugene 6 transfection reagent (Boehringer Mannheim, Germany) according to the manufacturer's instructions. In some experiments, 1 μ g pCMV-

GFP was cotransfected together with the FcR constructs. Cells were incubated at 37°C in a humidified CO₂ atmosphere for 48 hours before harvesting.

Ig-binding assays

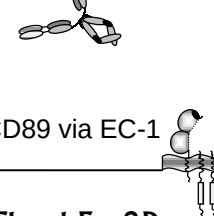
Uncoated magnetic M-450 Dynabeads (Dyna, Norway) were coated, according to the manufacturer's instructions, with either human serum IgA (hIgA) or bovine IgG2 (bIgG2), which were purified as previously described (7,32). Due to low transfection efficiency of some DNA constructs (see Results), transfected COS-1 cells were first enriched for those becoming positive for gene expression by cotransfection of the FcR and pCMV-GFP constructs. Experiments showed that most fluorescent (GFP⁺) cells had also taken up both plasmids, thus expressing the chimeric FcR together with GFP (see Results). Therefore, binding assays were performed as follows: 5 x 10⁴ GFP⁺ COS-1 cells (which had also been cotransfected with an FcR construct) were purified in a FACSVantage[®] cell sorter (Becton Dickinson) and mixed with Ig-coated Dynabeads in a final volume of 50 µl per well in V-bottomed microtiter plates. After a 15 min incubation at room temperature, the plate was spun at 50 g for 1 min and incubated for an additional 45 min at room temperature. Cells and beads were resuspended and examined for the presence of rosettes, using a combination of light and fluorescent microscopy, in a Nikon Eclipse E800 microscope. Rosettes were defined as GFP⁺ cells binding four or more Ig-coated beads and at least 200 GFP⁺ COS-1 cells were counted for each determination. For blocking studies, cells were incubated with either mAb My43 (50 µl culture supernatant) or CC-G24 (50 µl ascites fluid diluted 1:4) for 30 min at room temperature before the addition of Ig-coated beads.

Production and purification of recombinant soluble CD89

A cDNA that encodes a soluble form of FcαRI was expressed in Chinese hamster ovary cells by means of the pEE14 expression system (Lonza, UK) and the protein was isolated from the culture supernatant by affinity chromatography with Sepharose-bound human IgA (van Zandbergen, G., and C. van Kooten, unpublished data).

Monoclonal antibodies

The previously described FcαRI mAb's My43 (murine IgM), A3, A59, A62, and A77 (all murine IgG1) were used in this study (33,34). My43 and A62 were gifts from Dr. Li Shen (Dartmouth Medical School, Lebanon, NH) and Dr. Max Cooper (University of Alabama, Birmingham, AL), respectively. A77 was supplied by Medarex Europe (The Netherlands), and A3 and A59 were purchased from



Immunotech (France) and Research Diagnostics Inc. respectively. The bFc γ 2R mAb CC-G24 (murine IgM) was generated by immunizing mice with bFc γ 2R protein purified from cattle leukocytes, and the specificity was confirmed by staining COS-7 cells transfected with cDNA encoding the bFc γ 2R or bovine Fc γ RII (Howard, C.J., unpublished data). To obtain new Fc α RI mAbs, female BALB/C mice were immunized with purified soluble Fc α RI. Splenocytes isolated from immunized animals were fused with myeloma cells (SP20) in the presence of 50% polyethylene glycol. The cell suspension was diluted in IMDM supplemented with 10% FCS, hypoxanthine (100 μ mol), aminopterin (0.4 μ M), thymidine (16 μ M), 500pg/ml IL-6, 100U/liter penicillin, and 100 μ g/ml streptomycin. Cells producing antibodies to Fc α RI antibodies were subcloned by limiting dilution. Five clones producing mAb to Fc α RI were expanded and the specificity was determined by FACS[®] analysis (see below). To define the capacity of these new mAb to inhibit binding of IgA to Fc α RI, blocking studies were performed as follows. Fc α RI mAbs or control mAbs of the same isotype were diluted in FACS buffer and incubated together with Fc α RI-transfected IIA1.6 cells for 15 minutes at 4°C. Purified human serum IgA, which had previously been heat-aggregated for 1 hour at 63°C (algA), was then added for 1 h at 4°C. Cells were washed and bound IgA was detected by incubation with a goat anti-human IgA F(ab)₂-PE polyclonal antibody conjugate (Southern Biotechnology associates, Inc.) using FACS[®] analysis. Isotype control antibodies for murine IgG1 were purchased from Becton Dickinson, and those for murine IgM were provided by Dr. Robert Burns (Scottish Agricultural Science Agency, Edinburgh, UK).

FACS[®] analysis

Cells (5x10⁵) were washed twice with FACS buffer (PBS/0.5%BSA/0.02% azide) and incubated with either Fc α RI mAb (murine IgM or IgG1) culture supernatant or the appropriate isotype control supernatant for 1 h at 4°C. Cells were then washed twice with FACS buffer and incubated for 1 h with either goat anti-mouse (GAM) IgM-FITC conjugate (1:150 final dilution), or a GAM IgG1-PE conjugate (1:150 final dilution) (both from Southern Biotechnology Associates, Inc.). In experiments where GFP⁺ cells were analyzed for chimeric FcR expression a GAM IgG1 Tricolor secondary reagent (1:200 final dilution) (Caltag Labs.) was used. After washing twice with FACS buffer, cells were fixed in PBS-buffered 1% (wt/vol) paraformaldehyde at 4°C and analyzed on FACScan[®]. Data acquisition was conducted with Lysis II software (Becton Dickinson), and data analysis was performed using WinMDI software (available from The Scripps Research Institute, La Jolla, CA).

RESULTS

EC1 of CD89 and bFcγ2R mediate ligand binding

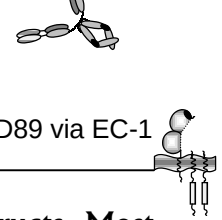
To map the ligand-binding domains of FcαRI and bFcγ2R, we generated five chimeric receptors as follows: hEC-bFcγ2R, consisting of the two EC domains of FcαRI fused to the transmembrane/cytoplasmic (TM/C) domain of bFcγ2R; bEC-FcαRI, the two bovine EC domains fused to TM/C domain of FcαRI; hEC1-bFcγ2R, the EC1 domain of FcαRI fused to EC2TM/C region of bFcγ2R; bEC1-FcαRI, the EC1 domain of bFcγ2R joined to EC2TM/C region of FcαRI; and bEC1⁽¹⁻⁵⁰⁾-FcαRI, the first 50 amino acids of bFcγ2R fused at isoleucine 50 to FcαRI (Figure 1).

	S1	S2	+1 EC1	
CD89	MDPKQTLLCLVLCGLGQRIQAQEGDFMPFISAKSSPVIPLDGSVKIQCC			29
bFcγ2R	*A*TLPA***G*SV*L*T*V*A*T**K*I*W*EP*S*V**GS**T*L**			29
CD89	AIREAYLTQLMIKNSTYREIGRRLKFWNETDPEFVIDHMDANKAGRYQC			79
bFcγ2R	GPPNTKSFS*NKEGD**PWN*HPS*EP*DKA--N*F*SNVREQQ****H*			77
		EC2		
CD89	QYRIGHYRFRYSDTLELVVTG-----LYGKPFSLADRLVLMPEGNISL			123
bFcγ2R	SHF**VNWSEP*EP*D*L*A*EEPAGR*RDR*S**VRSPSPA****VT*			127
CD89	TCSSAHIPDFRSLAKEGE---LSLP-QHQSGEHPANFSLGPVDLNVSG			167
bFcγ2R	L*Q*-GNRT*T*L*S***AAHRP*R*RS*D*D*WYQ*E***S**TSAHG*			176
		TM/C		
CD89	IYRCYGWYNRSPYLWSFSPNALELVVTD ^{SIHQDYTTQNLIRMAVAGLVLV}			217
bFcγ2R	T***RSLSTN***LSQ* <u>EP</u> *A*L*A*-----M*****GL*AS*L			221
CD89	ALLAILVENWHSHTALNKEASADVAEPSWSQMCQPGLTFARTPSVCK			265
bFcγ2R	L*GIL*CQAR*D*GGA-R**ARS			243

Figure 1. Alignment of amino acid sequences of FcαRI and bFcγ2R (Resp. X54154-Z37506). S1 exons are shown from the methionine initiation codons. Because the gene structure of bFcγ2R is unknown, intron-exon boundaries are shown only for FcαRI. Amino acids that link two FcαRI exons are underlined, and the exon designation is shown above the sequence. Note that exon-linking amino acids are conserved between FcαRI and bFcγ2R. The first 19 amino acids of both sequences are considered to represent NH₂-terminal signal peptides,

which are removed before cell surface expression. Thus, glutamine (Q) 22 is proposed to be the first amino acid of both mature proteins and is therefore designated +1. Isoleucine (I) is also underlined. Residues of bFcγ2R identical to those of FcαRI are designated (*), and gaps that have been inserted to line up the sequences are designated (-), (also see reference 7).

Together with wild-type FcαRI and bFcγ2R cDNAs, individual chimeric FcR constructs were transfected to COS-1 cells, and their cell surface expression was assessed by FACS[®] analysis and by a specific binding assay using Ig-coated beads (see Materials and Methods). Initial experiments revealed the transfection efficiency of individual constructs to be quite variable. The most efficient construct directed expression of FcαRI on the surface of ~30% of COS-1 cells, whereas the least efficient (bEC1-FcαRI) was expressed by only 3% of the transfectants (Figure 2A). Therefore, we developed a method to selectively enrich for transiently transfected cells by cotransfecting a plasmid that directed expression of GFP



(visualized by green fluorescence) together with the chimeric FcR constructs. Most GFP⁺ COS-1 cells cotransfected with Fc α RI were recognized by mAb A 62 (Figure 2B), and formed rosettes with hIgA-coated beads (Figure 2C).

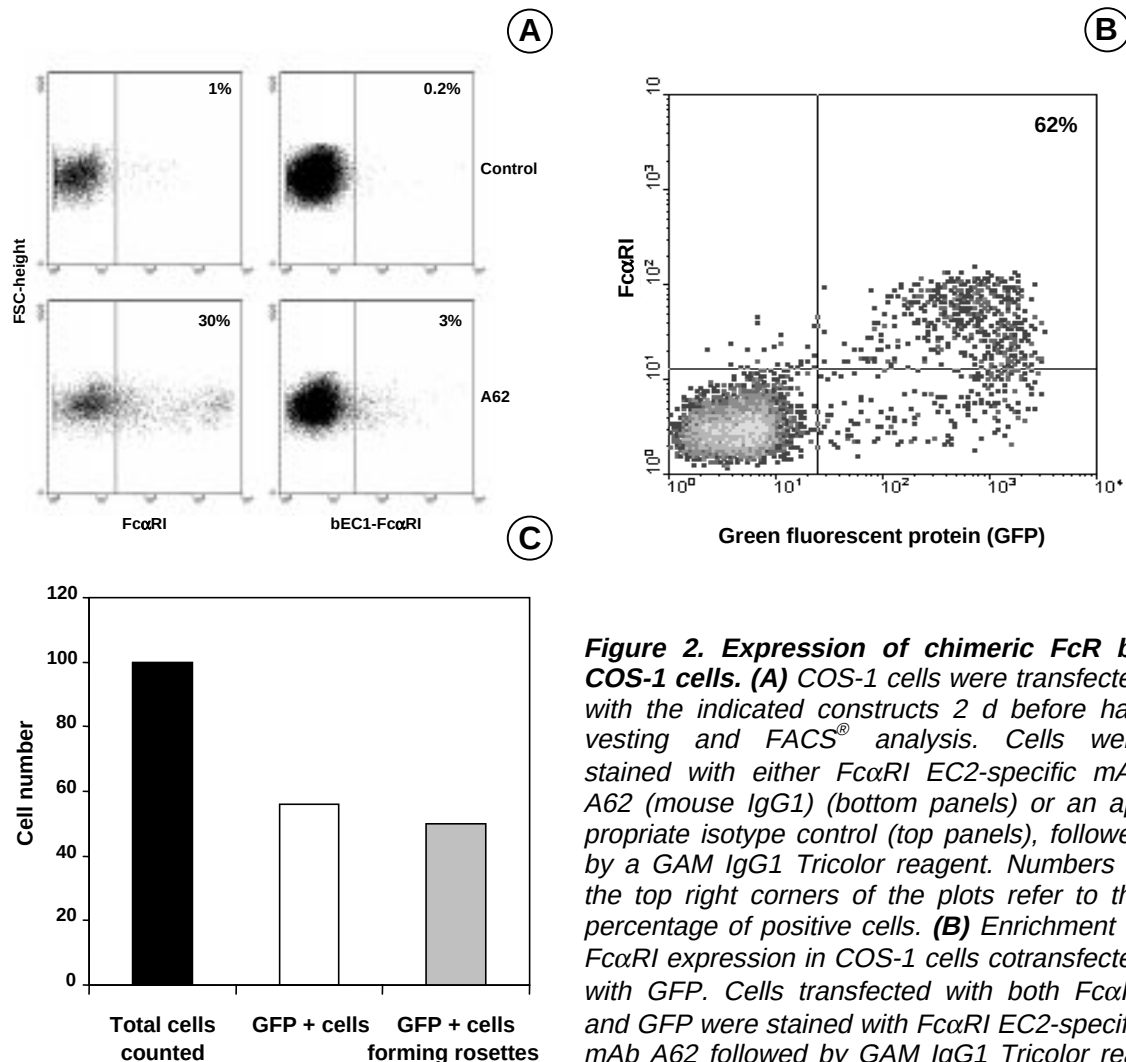


Figure 2. Expression of chimeric FcR by COS-1 cells. (A) COS-1 cells were transfected with the indicated constructs 2 d before harvesting and FACS[®] analysis. Cells were stained with either Fc α RI EC2-specific mAb A62 (mouse IgG1) (bottom panels) or an appropriate isotype control (top panels), followed by a GAM IgG1 Tricolor reagent. Numbers in the top right corners of the plots refer to the percentage of positive cells. (B) Enrichment of Fc α RI expression in COS-1 cells cotransfected with GFP. Cells transfected with both Fc α RI and GFP were stained with Fc α RI EC2-specific mAb A62 followed by GAM IgG1 Tricolor reagent and analyzed by FACS[®]. More than 60% of the GFP⁺ cells also express Fc α RI. (C) Rosette formation by COS-1 cells cotransfected

with both Fc α RI and GFP and exposed to hIgA-coated beads. Rosettes were quantified as specified in Materials and Methods. Black bar, total number of cells counted; white bar, total number of GFP⁺ cells assessed by fluorescent microscopy; gray bar, number of GFP⁺ cells forming rosettes. Results shown are representative of three separate experiments.

By this procedure we obtained an approximately twofold enrichment of chimeric FcR-expressing cells, which in the case of Fc α RI resulted in >60% of cells being reactive with Fc α RI mAb and further able to form rosettes with hIgA coated beads (Figure 2B and C). It should be noted that GFP⁻ COS-1 almost never formed rosettes, most likely because transfectants expressing Fc α RI alone accounted for only ~1% of the total cells (Figure 2B). We, furthermore, demonstrated the

specificity of our binding assay by using blocking mAbs specific for Fc α RI or bFc γ 2R to inhibit rosette formation (Figure 3). The finding that the inhibition obtained with My43 was only partial (~50%) was most likely explained by the

use of culture supernatant other than a higher concentration of purified antibody which was not available.

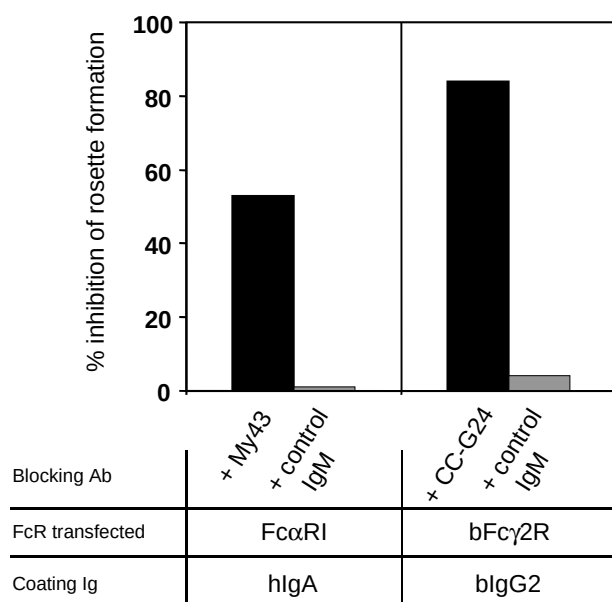
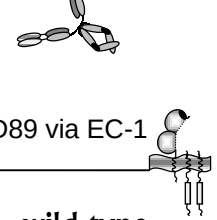


Figure 3. Specificity of the bead rosetting assays. COS-1 cells were co-transfected with the indicated FcR construct together with pCMV-GFP. GFP⁺ cells were purified as described in Materials and Methods and incubated with the relevant murine IgM-blocking mAb or an irrelevant murine IgM control mAb for 30 min before addition of Ig-coated beads. Results are shown, as percentages of inhibition of rosette formation when compared with transfectants that were not incubated with mAb before rosetting analysis. Results shown are representative of two experiments.

Fc α RI does not bind bIgG2, and bFc γ 2R does not bind hIgA (7); accordingly, we neither observed rosettes when hIgA-coated beads were mixed with bFc γ 2R transfectants, nor when bIgG2-coated beads were mixed with Fc α RI transfectants (Figure 4). Binding studies with COS-1 cells enriched for FcR expression as described above, showed not unexpectedly that wild-type Fc α RI and bFc γ 2R transfectants produced the highest levels of rosette formation with hIgA- and bIgG2-coated beads, respectively (Figure 4). Transfectants expressing chimeras coding for the entire EC portions of the receptors (hEC-bFc γ 2R and bEC-Fc α RI) bound their respective Ig-coated beads efficiently, although at a slightly lower level than their wild-type counterparts (Figure 4). The fact that the hEC1-bFc γ 2R chimera retained IgA-binding capacity demonstrated that the binding site of Fc α RI lies within the membrane-distal EC1 domain. Furthermore, because this chimera did not form rosettes with bIgG2-coated beads, our finding further suggested that the binding site for bIgG2 in bFc γ 2R was not located within the EC2 domain of this receptor. Conversely, the bEC1-Fc α RI chimera did form rosettes with bIgG2-coated beads, but not with beads coated with hIgA.

Altogether these results showed that, in common with Fc α RI, the ligand-binding site of bFc γ 2R appeared to lie within the EC1 domain. It should be noted, however, that the level of binding obtained with the two EC1 chimeras (hEC1-



bFcγ2R and bEC-FcαRI) was reduced when compared with the wild-type receptors, and the EC chimeras (hEC-bFcγ2R and bEC-FcαRI) (Figure 4).

Furthermore, to better localize the Ig-binding sites of these two receptors, a further chimera was constructed in which the first 50 amino acids of the EC1 domain were from bFcγ2R, while the remaining EC1 (49 amino acids) and the rest of the receptor were from FcαRI [bEC⁽¹⁻⁵⁰⁾-FcαRI]. Although this chimera was expressed at the cell surface and could be recognized by the majority of FcαRI mAb, it bound neither hIgA- nor bIgG2-coated beads.

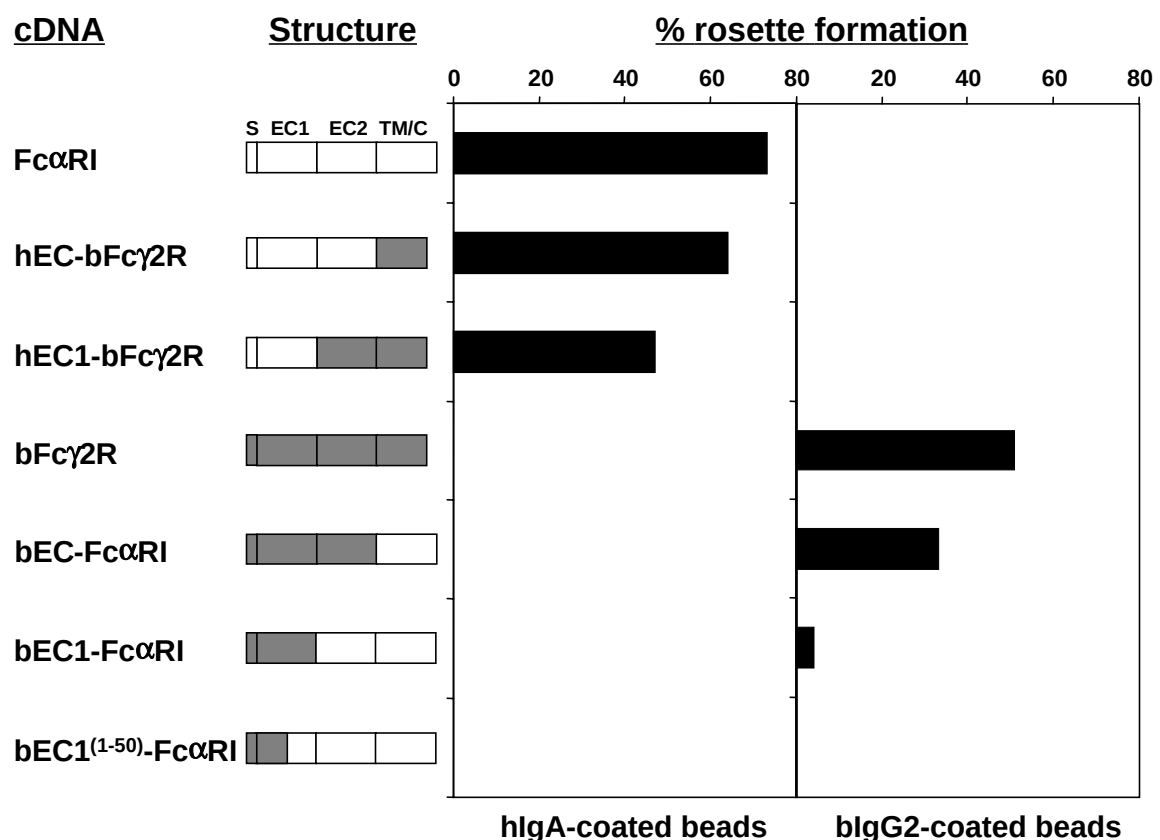


Figure 4. Rosette formation by FcR/GFP cotransfected COS-1 cells. Schematic representation of wild type or chimeric FcRs. Unshaded regions are derived from FcαRI, shaded regions from bFcγ2R. S, signal peptide; EC1, extracellular domain 1; EC2, extracellular domain 2; TM/C, transmembrane - cytoplasmic tail. GFP⁺ transfectants were purified from COS-1 cells cotransfected with GFP and FcR constructs as described. Ig-binding to FcRs coexpressed with GFP was assessed by rosetting with either hIgA- or bIgG2-coated beads. More than 200 cells were counted for each determination, and the number of cells binding four or more Ig-coated beads is expressed as percentage rosette formation. Results are representative of three separate experiments.

EC1-specific antibodies block CD89 binding of IgA

To confirm that the IgA-binding site of FcαRI lies within the EC1 domain, we mapped the specific epitopes for a number of blocking and nonblocking mAbs. Of

the previously described Fc α RI mAbs, only My43 (murine IgM) was able to block the binding of hIgA to Fc α RI (33). Four others (A3, A62, A59, and A77, all murine IgG1) did not inhibit binding (34). We also included a number of new Fc α RI mAbs, (2E6, 2D11, 7G4, 2H8, and 7D7, all murine IgG1), raised against a soluble form of Fc α RI. These mAbs were shown to be specific for Fc α RI by reaction with IIA1.6 cells expressing this receptor (Figure 5A).

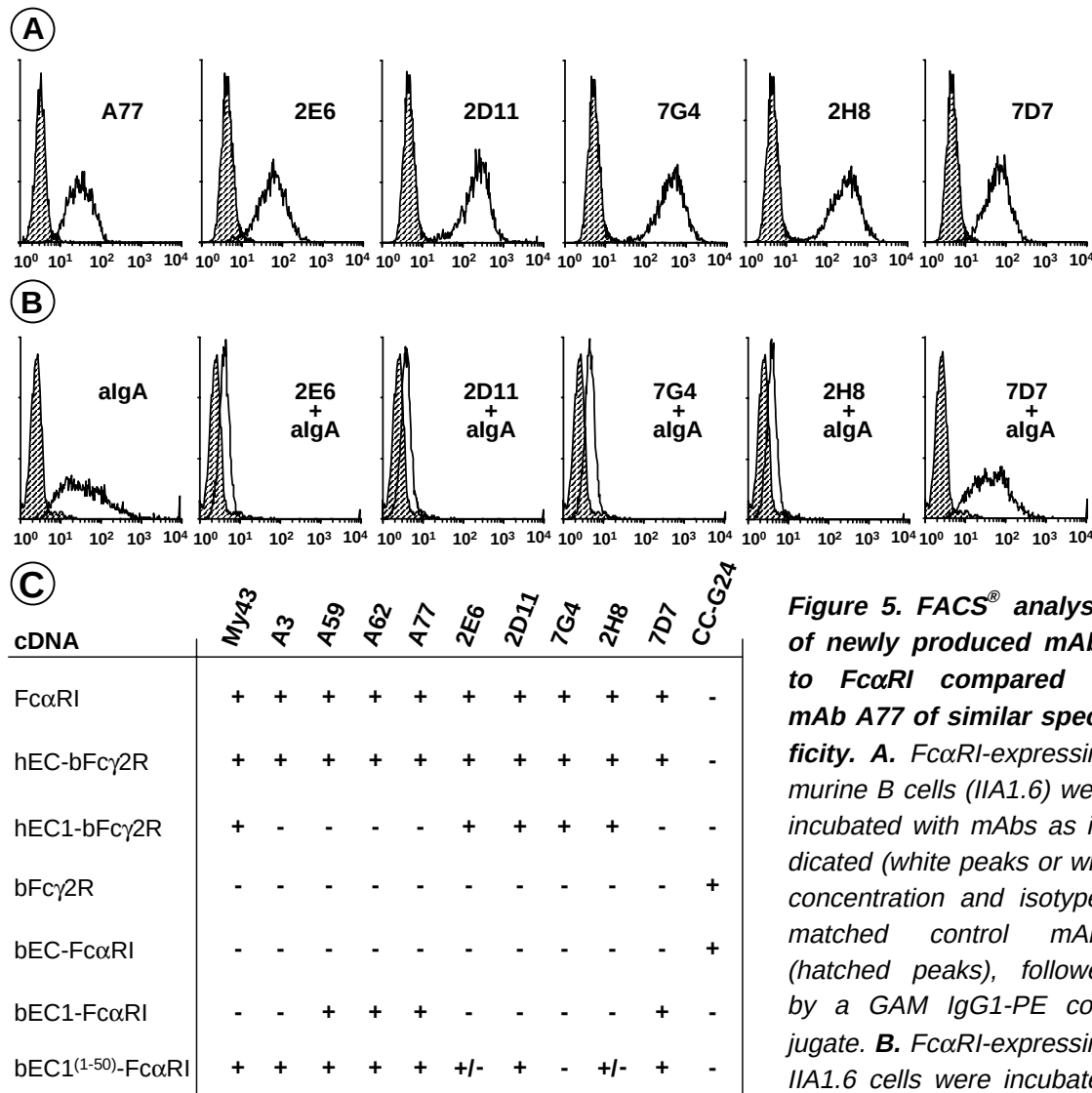
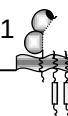


Figure 5. FACS[®] analysis of newly produced mAbs to Fc α RI compared to mAb A77 of similar specificity. **A.** Fc α RI-expressing murine B cells (IIA1.6) were incubated with mAbs as indicated (white peaks or with concentration and isotype-matched control mAbs (hatched peaks), followed by a GAM IgG1-PE conjugate. **B.** Fc α RI-expressing IIA1.6 cells were incubated without (left panel) or with

mAbs as indicated, followed by heat-aggregated hIgA (algA). After 1 h at 4°C, cells were washed and bound hIgA was detected by incubation with goat anti-human IgA F(ab)₂-PE conjugate (white peaks). Cells incubated with only the secondary reagent served as negative controls (hatched peaks). **C.** Reactivity of chimeras (left) with a panel of Fc α RI and bFc γ 2R mAb (top) as measured by FACS[®] analysis. Binding was graded as follows: +, strong binding; +/-, weak binding; -, no binding.

They were next assayed for ability to block binding of heat-aggregated hIgA to Fc α RI: mAbs 2E6, 2D11, 7G4, and 2H8 produced such inhibition while 7D7 did



not (Figure 5B). Accordingly, we presumed that the blocking mAbs would prove to be EC1- specific, whereas the nonblocking ones would be EC2 specific. To test this hypothesis, we screened the reactivity of all mAbs against the panel of chimeric FcR expressed in COS-1 cells by FACS[®] analysis. Indeed all mAbs capable of blocking the binding of heat-aggregated hIgA to Fc α RI (Figure 5B), mapped to the EC1 domain (Figure 5C).

Also, all nonblocking mAbs were directed against the EC2 domain, except for mAb A3 that apparently recognized an epitope depending on parts of both domains. Unfortunately, because only one mAb against bFc γ 2R was available, a similar detailed study could not be performed for this receptor. We showed that the mAb CC-G24 only recognized wild-type bFc γ 2R and the bEC-Fc α RI chimera (Figure 5C). Thus, like mAb A3, it is most likely directed against a conformational epitope depending on both EC1 and EC2.

DISCUSSION

By means of a panel of chimeric FcRs, we identified conclusively for the first time the ligand-binding sites of Fc α RI and bFc γ 2R. Surprisingly, these sites were found to be located in the EC1 domains. Fc α RI and bFc γ 2R are highly homologous both at the protein and nucleotide level (41% and 56% identity, respectively), but show much less homology with other human and bovine FcRs (7). This suggests that Fc α RI and bFc γ 2R evolved from a common ancestral gene (also shared by KIR, ILT, MIR, LIR, LAIR-1, HM18, PIR, and gp49B1 genes) and not shared by other human or bovine FcRs (7). Mapping of the bFc γ 2R gene to bovine chromosome 18, which corresponds to human chromosome 19, further supports this notion (35).

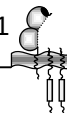
Our finding that the Ig-binding sites of both Fc α RI and bFc γ 2R are located within their EC1 domains, was based on the fact that rosetting with Ig-coated beads was only seen for the corresponding Fc α RI or bFc γ 2R EC1 chimera. In both cases, however, a reduction in binding activity was seen compared with that obtained for the wild-type receptors and for the comparable EC chimeras (Figure 4). Although these differences in part may be attributed to the expression levels of individual constructs (especially for the bEC1- Fc α RI chimera, see above), it is also possible that the EC2 domains and membrane-proximal regions of the receptors contribute either directly (by forming “secondary” contact sides) or indirectly (by preserving three-dimensional structure) to the affinity and stability of the ligand interactions. This would be analogous to the activity of other two- domain FcRs, namely Fc γ RII, Fc γ RIII, and Fc ϵ RI in which the ligand-binding sites are located in the membrane-

proximal EC2 domains, whereas structures within the EC1 domains contribute to the binding process (29,30).

It should also be noted that the region of hIgA interacting with Fc α RI has recently been mapped to the C α 2/C α 3 boundary (36). This is in contrast to the region of human IgG responsible for interaction with Fc γ Rs, which is proposed to lie much closer to the hinge (30). Therefore, in terms of the evolution of Ig/FcR interactions, it should be interesting to map the region of bIgG2 that binds to bFc γ 2R. Because recombinant bIgG2 is available, experiments can readily be designed to determine whether this region lies close to the hinge region as in human IgG or in a position analogous to that of hIgA (37).

To substantiate our observation that IgA binds to the EC1 domain of Fc α RI, we mapped the epitopes of a panel of blocking and nonblocking anti-Fc α RI mAbs that bound equally to the EC parts of wild-type Fc α RI and the hEC-Fc γ 2R chimera. The nonblocking mAbs (A59, A62, A77, and 7D7) were shown to react with the membrane-proximal EC2 domain because they bound only to wild-type Fc α RI and the hEC-bFc γ 2R, bEC1-Fc α RI, and bEC1⁽¹⁻⁵⁰⁾Fc α RI chimeras. The only exception was the nonblocking mAb A3 that bound only to wild-type Fc α RI and the hEC-bFc γ 2R, and bEC1⁽¹⁻⁵⁰⁾-Fc α RI chimeras, suggesting that its epitope is conformational and depends on regions of both EC1 and EC2 (similar to the bFc γ 2R mAb CC-G24, see Results section). In contrast, all blocking mAbs (My43, 2E6, 2D11, 7G4, and 2H8) were shown to react with the EC1 domain of Fc α RI because their binding activity was retained with the hEC1-bFc γ 2R chimera. The epitopes recognized by My43 and 2D11 were further localized to the region of EC1 directly adjacent to EC2, because they were shown to bind the bEC1⁽¹⁻⁵⁰⁾-Fc α RI chimera. 2E6 and 2H8 on the other hand, bound only weakly to this chimera, whereas 7G4 did not bind at all. Similar mapping studies with blocking mAbs have previously been used to localize the IgG-binding sites of Fc γ RII and Fc γ RIII to their EC2 domains (25,27).

A number of Fc α RI mRNAs have been isolated and shown to encode splice variants of the receptor (38-41). One such report described cell surface expression of an Fc α RI variant that lacked the complete EC2 domain, and suggested the EC1 domain to be involved in hIgA binding (40). However, in contrast to our results, mAb My43 was proposed to react with EC2, and mAb A59 with EC1. We believe that observation to be spurious either due to incorrect cell surface expression of the splice variant and/or aberrant receptor structure caused by lack of the complete EC2 domain. This possibility was supported by our attempts to express various Fc α RI splice variants in COS cells with no success (38). Additionally, chimeras constructed between Fc α RI and Fc γ RII were not expressed



efficiently (Morton, H.C., and J.G.J. van de Winkel, unpublished observations), possibly reflecting a degree of structural incompatibility between these two FcRs. In fact, our unsuccessful experience with those approaches led us to construct chimeras between Fc α RI and bFc γ 2R as reported here, because their levels of homology (and hence presumably their overall structure) are more similar than for other FcRs. Thus swapping of highly homologous regions should have minimal affect on the overall structural integrity of the resultant chimeras. Therefore, we feel that the present approach is more physiological than previous attempts to this end.

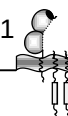
The surprising difference seen between the ligand-binding sites of Fc α RI and bFc γ 2R versus those of other leukocyte Fc γ Rs and Fc ϵ RI may have interesting implications in terms of Ig interactions. As mentioned above, this disparity could simply reflect the proposed evolution of Fc α RI and bFc γ 2R from an ancestral gene distinct from that giving rise to other Fc γ Rs and Fc ϵ RI. This notion is supported by the observation that residues within the membrane-distal domain of two KIR proteins determine their ability to bind to their respective ligands, the two groups of HLA-C allotypes (42). Moreover, due to their high levels of homology, the three-dimensional structure of Fc α RI and bFc γ 2R may more closely resemble that of the KIR proteins than that of more distantly related FcRs (43). Indeed, more detailed mutational analysis, directed by modeling studies using the recently published three-dimensional structure of the p58 KIR as a template for the protein backbones of Fc α RI and bFc γ 2R are currently underway in our laboratory to further localize the Ig-binding sites within these two receptors.

An alternative evolutionary explanation possibly applicable at least for Fc α RI might be that its ligand-binding site developed to ensure interaction with all molecular forms of IgA: monomeric IgA, dimeric IgA (including J-chain), and secretory IgA (including J-chain and secretory component). Fc α RI is reported to bind all these ligand variants (44,45). Therefore, because the site of interaction with Fc α RI at the C α 2/C α 3 boundary appears to be accessible to the receptor in all these forms of IgA, Fc α RI could have evolved to accomplish this interaction via its EC1 domain to avoid potential problems of steric hindrance of a more membrane-proximal binding site in relation to large IgA polymers.

In conclusion, we have shown that the closely related Fc α RI and bFc γ 2R bind their ligands via sites located in their membrane-proximal EC1 domains. The difference in the Ig-binding sites of these two receptors versus other leukocyte Fc γ Rs and Fc ϵ RI, may reflect the proposed divergent evolutionary pathway from a distinct genetic precursor, or (at least in the case of Fc α RI) a specific adaptation for efficient interaction with large molecular forms of IgA.

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